EFFECTIVENESS OF FUNGICIDAL CHEMICALS IN PREVENTING THE GROWTH OF TRICHOPHYTON INTERDIGITALE AND EPIDERMOPHYTON FLOCCOSUM IN SHOE LEATHER

By Dorothea E. Klemme, *formerly textile chemist*, and Agnes C. Baldwin, *formerly bacteriologist, Bureau of Human Nutrition and Home Economics, Agricultural Research Administration*[1]

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INTRODUCTION

The studies reported here were made to determine the fungicidal effectiveness against *Trichophyton interdigitale* and *Epidermophyton floccosum (3, pp. 209, 210)*[2] of various chemical compounds when they are incorporated in shoe dubbing and applied to leather. The investigation was part of wartime research. Although the goal was the control of foot infections caused by these dermatophytes in the leather of shoes, the end of the war terminated this work before the toxicity of the chemical-dubbing mixtures had been determined. Therefore, until such toxicity tests are made by some other agency, the treatments that were found to inhibit effectively the growth of the fungi in leather should not be applied to materials which are to be made into shoes.

[1] Grateful acknowledgment is made to E. L. Wallace and J. R. Kanagy, Leather Section, National Bureau of Standards for their suggestions and for the leather and shoe dubbing used.

Appreciation is expressed to Frances Jane Stouffer, formerly bacteriologist, Bureau of Human Nutrition and Home Economics, for assistance in checking results of some of the experimental work.

[2] Italic numbers in parentheses refer to Literature Cited, p. 18.

LITERATURE REVIEW

The trouble caused by the growth of mold on leather during the tanning processes led to research in which various chemicals were used to inhibit the growth of fungi *(1, 7, 10)*. Some compounds which proved effective were beta-naphthol, paranitrophenol, phenyl mercurial salts, and chlorophenol derivatives.

In 1944, Lollar *(8, 9)* published the results of an extensive study in which he used some of the same chemicals that earlier investigators had found were fungicidal, along with many others, in the development of mildew-resistant treatments for leather. He found that paranitrophenol, parachlorometaxylenol, salicylanilide, parachlorophenol, tetrachlorophenol, 2,4,5-trichlorophenol, 2,2'-dihydroxy-5,5'-dichlorodiphenylmethane, and 2-mercaptobenzothiazole effectively inhibited the growth of mildew-producing organisms on leather.

Greene and Lollar *(6)* reported that incorporating preservatives in the dubbing applied to leather as a dressing, protected it against mold and mildew. These authors pointed out that by putting the protective agent in the dubbing and rubbing it on the leather the preservative was on the surface where molds first start to grow.

The success which various investigators had in using chemicals for inhibiting growth of mildew-producing fungi on leather suggested that fungicidal chemicals when incorporated in shoe dubbing and applied to leather, would inhibit growth of *Trichophyton interdigitale* and *Epidermophyton floccosum*, two dermatophytes commonly associated with athlete's foot.

MATERIALS

Eighty-one chemicals and combinations of chemicals known to be germicidal or to have properties similar to those of proved fungicides were investigated. These substances belonged, for the most part, to the following classes of compounds: Substituted phenols and phenol derivatives, amines, acetate esters, organic mercurials, alkyl ammonium halides.

Chrome-retanned leather, the kind ordinarily used in shoe uppers, was selected for the work done by the Bureau of Human Nutrition and Home Economics since it met the two essential requirements, namely, it was nontoxic to the test organisms and it could be steam sterilized. Fulton, Gibbons, and Moore *(4)* have also found that vegetable-tanned leather, when new, had a

fungicidal effect upon certain dermatophytes. It, therefore, was unsuitable for this study. Also, whereas steam sterilization hardened the vegetable-tanned leather, chrome-tanned leather could be sterilized by intermittent steaming, thus killing any contaminating fungi which would outgrow the organisms to such an extent that the viable dermatophytes might easily be overlooked during visual examination.

The test organisms, *Trichophyton interdigitale* and *Epidermophyton floccosum*, were transfers from the American Type Culture Collection, numbers 7124 and 4808, respectively.

LABORATORY PROCEDURE

Routine Bacteriological Methods

Since fungicides frequently show specificity for different organisms, preliminary experiments were conducted to determine which chemicals were effective[3] against *Trichophyton interdigitale* and *Epidermophyton floccosum*. The procedures used for eliminating ineffective chemicals were adaptations of those described by Ruehle and Brewer *(11)*; namely, the wet filter-paper, the dry filter-paper, and the agar-plate methods, the particular one depending upon the type of substance under investigation.

The compounds effective in preventing growth of the two fungi, even to a slight extent in the preliminary tests were studied further.

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[3] See table 1 for a list of substances effective in preliminary investigations. Those that failed to inhibit growths of the two dermatophytes in preliminary experiments were: (1) mono-n-octadecyl amine acetate (25 percent), mono-n-hexadecyl amine acetate (25 percent), and mono-n-octadecenyl amine acetate (50 percent); (2) mono-n-dodecyl amine acetate; (3) mono-n-hexadecyl amine acetate; (4) parachloro-sym.-metadimethylhydroxy benzene and paratertiaryamylhydroxy benzene; (5) sodium salt of orthophenylphenol; (6) sodium salt of chloro-2-phenylphenol; (7) sodium salt of 2-chloro-4-phenylphenol; (8) sodium salt of pentachlorophenol; (9) chloro-2-phenylphenol; (10) 2-chloro-4-phenylphenol; (11) a commercial mixture of coal tar hydrocarbons, phenols, and soap; (12) chloramine-T; (13) quasia; (14) bismuth derivative of campho-carboxylic acid; (15) bismuth formic iodide; (16) metacresylacetate; (17) 2-chloro-6-nitrotoluene; (18) 6-nitrotrichlorotoluene; (19) hexametacresol; (20) 8-hydroxyquinoline sulfate; (21) methyl parahydroxybenzoate; (22) naphthenic acid; (23) sodium salicylate; (24) thymol iodide; (25) commercial mixture of sodium perborate, hydroxyquinoline, aluminum silicate, sodium borate and boric acid; (26) mixture of alkyl-dimethyl-benzyl-ammonium chlorides; (27) alpha-alphadimethyl-alpha-carbobutoxy-dihydro-gamma-pyrone; (28) phenothiazine and ferric chloride; (29) cottonseed oil; (30) soybean oil; (31) peanut oil; and (32) linseed oil.

Preliminary Single-Sample Tests

Preparation of chemical-dubbing mixture.--Various concentrations of each chemical were incorporated in a shoe dressing consisting of 50 percent tallow, 40 percent neatsfoot oil, 9 percent amorphous wax, and 1 percent aluminum tristearate which is the same as that used by the United States Army for service shoes *(2)*. To prepare the chemical-dubbing mixture, the dubbing was weighed into flasks and sterilized by autoclaving at 15 pounds for 20 minutes. The desired amount of the particular chemical under investigation was then added and mixed thoroughly with the dressing before it hardened. Several concentrations of the chemical were tested in order to determine the minimum percentage that would give inhibition. If a compound was not effective in a concentration of 6 percent or less, it was considered impractical for the purpose of this study. However, in some instances, the highest percentage that could be tested was less than 6 percent because of the limited solubility of the chemicals in the dubbing.

Throughout this investigation sterile technique was used, except in cases where this was unnecessary, as for example in the weighing of the chemicals in full strength. Even in these cases precautions were taken not to contaminate the dubbing through careless handling.

Preparation of the leather.--Two sizes of leather samples were cut. The larger, 3 by 4.5 cm., were used in testing the effectiveness of the various compounds in inhibiting the growth of *T. interdigitale* and *E. floccosum* on leather; the smaller, 2 by 3 cm., in determining the effects of water leaching and of storage upon the inhibiting action of effective dubbing-chemical mixtures on the leather. The samples were sterilized by intermittent steaming at 100° ± 0.5° C. for exactly 20 minutes on each of 3 successive days. The steaming was done at approximately 24-hour intervals. This treatment destroyed contaminating organisms and, except in occasional instances, caused practically no stiffening of the leather. Longer periods of steaming, such as the 30 minutes usually recommended for this type of sterilization, were unnecessary and caused much more hardening of the leather.

Applying chemical-dubbing mixture to leather.--The chemical-dubbing mixture was brushed onto the sterilized leather samples with a medium-sized, water-color brush, this method having been found more satisfactory than either rubbing the mixture into

the leather with a flattened glass rod or dipping the samples into some of the melted material. To insure good absorption of the dressing, the samples and sufficient dubbing mixture to treat each set of specimens were warmed, before brushing, in separate petri dishes for 15 minutes in an oven set at 50° to 60° C.

Starting at one end of the sample and brushing crosswise with approximately 30 strokes distributed evenly over the area to be treated, as much of the dubbing mixture as would be absorbed was put on the leather. Any unabsorbed excess was removed with the brush. Only one-half of each 3- by 4.5-cm. sample was brushed, the other half being left unbrushed in order to determine whether or not the chemical diffused, to any appreciable extent, from the treated portion of the leather into the untreated part. To obtain information concerning the diffusibility of the chemical through the leather, one sample was brushed on only the finished side of the leather. A duplicate specimen was treated on both the finished and the flesh side, the treatments, in every case, being applied to the same end of the sample.

The chemical-dubbing mixture was applied seven times at daily intervals. After each application of the dressing each sample was put in a clean, sterile petri dish, returned to the oven for 10 minutes to facilitate absorption, then left overnight at room temperature. After the last application the samples were inoculated with the fungi.

The small, 2- by 3-cm. pieces of leather for the leaching and storage tests were prepared in exactly the same way as the larger samples, except that the entire surface of the specimens was brushed.

Inoculation and incubation of treated samples.--Prior to inoculating the leather samples, plates of growth of the two test fungi, *T. interdigitale* and *E. floccosum*, were prepared as follows: Cultures were grown from 5 to 7 days at 37° C. on Sabouraud's agar slants in 18- by 150-ml. tubes containing about 10 ml. of medium[4]. To make a suspension of this growth for seeding plates, approximately 5 ml. of sterile distilled water was added to each tube and the growth gently worked free with a stiff wire inoculating loop. For each plate, 0.5 ml. of the seeding suspension of one of the fungi was mixed with 15 ml. of melted and cooled Sabouraud's agar, then poured into a 100 mm.

[4] This medium consisted of 10 gm. of peptone, 40 gm. of dextrose, and 20 gm. of agar per 1,000 ml. distilled water.

petri dish to harden. The seeded plates were incubated at 37° C. for 4 to 5 days after which time they were covered with a thick, even mat of creamy white fungus growth and were ready for inoculating the leather samples.

Two of the larger leather specimens, both treated with the same dubbing mixture, were laid flesh side down on an actively growing culture with the untreated ends of the sample toward the center of the dish. This separated as widely as possible the treated areas so that any clearing of the seeded agar caused by the fungicide on the leather could be readily noted. The smaller samples were inoculated in the same manner as the larger ones, and in order to leave sufficient space around each piece for observing zones of inhibition, no more than three of the little pieces were placed on a plate. The leather samples were exposed to the fungus growth for 2 days at 37° C., the temperature at which all cultures and inoculated samples were incubated throughout this study.

After inoculation, the pieces of leather were transferred, flesh side up, from the plates of fungus growth to glass fabric wicks in culture chambers containing Sabouraud's broth and incubated for a 30-day period. The culture chambers were 16-ounce, square bottles prepared as described by Greathouse, Klemme, and Barker *(5)* for testing fabric deterioration. Twenty-five milliliters of broth were put into each bottle and, in order to maintain the volume of medium between 20 and 25 ml. during incubation, small quantities of sterile distilled water were added at 10-day intervals. After 5, 10, 15, and 30 days the plates were observed for growth of the test fungi on the leather samples.

Effect of leaching and storage.--Those chemical-dubbing mixtures that prevented growth of *T. interdigitale* and *E. floccosum* on the leather samples were studied further to determine their ability to retain these inhibiting properties after water leaching and after storage for 2 months. Triplicate sets of the 2- by 3-cm. samples were sterilized. One set was treated on the finished side and one on both sides, according to the procedure described on page 4. The third set was left untreated to serve as a growth control for the fungi.

To leach the samples, two similarly treated pieces and the corresponding controls were placed separately in 16-ounce, wide-mouthed bottles, each containing 300 ml. of sterile distilled water. At intervals of 2, 2, 3, and 18 hours the samples were transferred to bottles of fresh water. They were left in

the fifth rinse for 6 hours thus making a total leaching period of 31 hours. The leached samples were then drained and inoculated by placing them, flesh side down, on viable plates of test fungi, separate plates being used for the treated and control specimens. After 2 days, the pieces of leather were transferred to culture chambers. Three samples, all of which were inoculated with the same fungus, were put in each bottle. One of the specimens was untreated; two were treated, one on the finished side and the other on both sides, with the same chemical-dubbing mixture. The samples were incubated for 30 days during which time they were observed at intervals for growth of the fungi.

To determine the effect of storage upon the inhibiting action of the fungicides, samples treated with each of the chemical-dubbing mixtures were placed in sterile petri dishes, wrapped individually in paper, and stored in the dark for 2 months. They were then inoculated by grafting and incubated as for the leaching test. Untreated samples stored at the same time served as controls for the growth of the fungi.

Verification of Preliminary Results by Replicate Tests

In the preliminary tests which screened out ineffective chemicals, a single sample of leather treated on only the finished side and one treated on both sides were used for each concentration of a given chemical-dubbing mixture for each fungus. In subsequent tests of the eight chemicals which the exploratory investigations showed had a very good inhibiting action, five replicate samples were used. The replicates for the three tests--the inhibiting action, the leaching, and the storage--were all treated simultaneously and the experiments previously described, repeated.

Differential Tests for Fungicidal-Fungistatic Action

When a chemical showed good inhibition against the two test fungi on leather and retained all or most of this property after leaching and storage, further experiments were conducted to determine whether the inhibiting action was fungistatic or fungicidal. For this purpose, two sets of five replicate samples, 1.5 by 2.5 cm., were prepared for each chemical-dubbing mixture for each organism. All specimens were sterilized; one set was treated on both sides with the fungicide dressing, the other brushed on only the finished side. In both sets, the

TABLE 3.--*Effectiveness of eight chemicals incorporated in shoe dubbing a*
Epidermophyton floccosum before and after leaching a

Treatment No.[1]	Chemical	Concentrations in dubbing	R No.	Before leaching or storage								
				Trichophyton interdigitale				*Epidermophyton floccosum*				
				One side		Two sides		One side		Two sides		One side
		Percent		*T*	*U*	*T*	*U*	*T*	*U*	*T*	*U*	
3c	Phenylmercuri-9-acetoxy-12-octadecanoic.acid.	2.0	1	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0
			3	0	0	0	tr	0	tr	0	tr	0
			4	0	0	0	0	0	0	0	0	0
			5	0	0	0	tr	0	0	0	tr	0
6b	Sodium ethylmercurithio-salicylate.	.15	1	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0
			3	0	0	0	0	0	0	0	0	0
			4	0	0	0	0	0	0	0	0	0
			5	0	0	0	0	0	0	0	0	0
6d	----------do----------	25	1	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0
			3	0	0	0	0	0	0	0	0	0
			4	0	0	0	0	0	0	0	0	0
			5	0	0	0	0	0	0	0	0	0
8	2,4,5-trichlorophenol-------	2.0	1	0	0	0	0	0	1+	0	2+	0
			2	0	0	0	0	0	0	0	2+	0
			3	0	0	0	0	0	2+	0	0	0
			4	0	1+	0	0	0	0	0	0	0
			5	0	tr	0	2+	0	0	0	0	0
9	2,3,4,6-tetrachlorophenol---	5.0	1	0	tr	0	0	0	0	0	tr	0
			2	0	0	0	0	0	0	0	0	0
			3	0	0	0	0	0	0	0	0	0
			4	0	0	0	tr	0	tr	0	0	0
			5	0	0	0	0	0	0	0	0	0
12a	Paranitrophenol------------	3.0	1	0	0	0	0	0	0	0	0	0
			2	0	tr	0	tr	0	0	0	0	tr
			3	0	tr	0	0	0	0	0	0	tr
			4	0	0	0	0	0	0	0	0	tr
			5	0	0	0	0	0	0	0	0	0
12b	----------do----------	5.0	1	0	tr	0	tr	2[2]0	2[2]0	2[2]0	2[2]0	0
			2	0	tr	0	0	0	0	0	0	0
			3	0	tr	0	tr	0	0	0	0	0
			4	0	0	0	0	0	0	0	0	tr
			5	0	0	0	0	0	0	0	0	0
14c	Parachlorometacresol--------	6.0	1	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0
			3	0	0	0	0	0	0	0	0	0
			4	0	0	0	0	0	0	0	0	0
			5	0	2+	0	0	0	0	0	0	0
15c	Parachlorometaxylenol-------	6.0	1	0	tr	0	0	0	0	0	0	tr
			2	0	0	0	0	0	0	0	0	0
			3	0	tr	0	0	0	0	0	0	tr
			4	0	tr	0	tr	0	tr	0	0	tr
			5	0	0	0	0	0	0	0	0	4+
17b	2,3-dichloronaphtha-quinone-1,4.	6.0	1	0	0	0	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0
			3	0	0	0	tr	0	tr	0	tr	0
			4	0	0	0	0	0	0	0	0	0
			5	0	0	0	tr	0	0	0	tr	0

[1] Treatment numbers correspond to those used in tables 1 and 2.

[2] Reading at the end of 15 days' incubation (bottle broken).

Abbreviations: R = replicate; T = treated; U = untreated; tr = trace.

lied to leather in preventing growth of Trichophyton interdigitale and er 2 months' storage when tested in replicate

After leaching				After storage					
ophyton igitale	*Epidermophyton floccosum*			*Trichophyton interdigitale*			*Epidermophyton floccosum*		
Untreated controls	One side	Two sides	Untreated controls	One side	Two sides	Untreated controls	One side	Two sides	Untreated controls
0	0	0	tr	0	0	0	0	0	2+
tr	0	0	tr	0	0	2+	0	0	2+
tr	0	0	tr	0	0	0	0	0	2+
0	0	0	2+	0	0	2+	tr	0	2+
0	tr	0	tr	0	0	0	0	0	0
2+	0	0	1+	0	0	0	0	0	0
2+	0	0	2+	0	0	0	0	0	0
1+	0	0	2+	0	0	2+	0	0	0
1+	0	0	1+	0	0	tr	0	0	0
2+	0	0	3+	0	0	2+	0	0	0
tr	0	0	0	0	0	1+	0	0	tr
0	0	0	tr	0	0	2+	0	0	tr
tr	0	0	tr	0	0	tr	0	0	tr
0	0	0	tr	0	0	1+	0	0	tr
tr	0	0	tr	0	0	1+	0	0	tr
1+	0	0	0	0	0	2+	0	0	2+
2+	0	0	4+	0	0	2+	0	0	tr
4+	0	0	4+	0	0	2+	0	0	2+
4+	0	0	4+	0	0	tr	0	0	2+
4+	0	0	4+	0	0	3+	0	0	2+
3+	0	0	1+	0	0	2+	0	0	tr
3+	0	0	2+	0	0	1+	0	0	0
2+	0	0	2+	0	0	1+	0	0	0
2+	0	0	1+	0	0	tr	0	0	2+
3+	0	0	3+	0	0	2+	0	0	2+
4+	0	0	tr	0	0	4+	0	0	tr
4+	0	0	tr	tr	tr	4+	0	0	tr
4+	0	0	tr	0	0	4+	0	0	tr
4+	0	0	tr	0	0	3+	0	0	tr
4+	0	0	1+	0	0	4+	0	0	tr
2+	0	0	tr	0	0	tr	0	0	0
tr	0	0	tr	0	0	0	0	0	0
2+	0	0	2+	0	0	tr	0	0	0
2+	0	0	tr	0	0	2+	0	0	0
2+	0	0	tr	0	0	3+	0	0	4+
tr	0	0	tr	0	0	tr	0	0	0
1+	0	0	tr	0	0	tr	0	0	0
0	0	0	tr	0	0	2+	0	0	0
3+	0	0	tr	0	0	tr	0	0	0
1+	0	0	2+	0	0	tr	0	0	tr
2+	tr	0	3+	0	0	0	0	0	1+
2+	0	0	4+	0	0	tr	0	0	1+
2+	tr	0	4+	0	0	1+	0	0	2+
2+	tr	0	4+	0	0	tr	0	0	1+
3+	tr	0	4+	0	0	1+	0	0	1+
1+	0	0	tr	0	0	2+	0	0	2+
tr	0	0	tr	0	0	2+	0	0	2+
tr	0	0	tr	0	0	2+	0	0	2+
2+	0	0	3+	0	0	2+	0	0	2+
1+	tr	0	tr	0	0	3+	0	0	1+

TABLE 3.--*Effectiveness of eight chemicals incorporated in shoe dubbing and applied to leather in preventing growth of* Trichophyton interdigitale *and* Epidermophyton floccosum *before and after leaching and after 2 months' storage when tested in replicate*

Treatment No.[1]	Chemical	Concentrations in dubbing	R No.	Before leaching or storage								After leaching						After storage					
				Trichophyton interdigitale				*Epidermophyton floccosum*				*Trichophyton interdigitale*			*Epidermophyton floccosum*			*Trichophyton interdigitale*			*Epidermophyton floccosum*		
				One side		Two sides		One side		Two sides		One side	Two sides	Untreated controls	One side	Two sides	Untreated controls	One side	Two sides	Untreated controls	One side	Two sides	Untreated controls
		Percent		*T*	*U*	*T*	*U*	*T*	*U*	*T*	*U*												
3c	Phenylmercuri-9-acetoxy-12-octadecanoic acid.	2.0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	tr	0	0	0	0	0	2+
			2	0	0	0	0	0	0	0	0	0	0	tr	0	0	tr	0	0	2+	0	0	2+
			3	0	0	0	tr	0	tr	0	tr	0	0	tr	0	0	tr	0	0	0	0	0	2+
			4	0	0	0	0	0	0	0	0	0	0	0	0	0	2+	0	0	2+	tr	0	2+
			5	0	0	0	tr	0	0	0	tr	0	0	0	tr	0	tr	0	0	0	0	0	0
6b	Sodium ethylmercurithiosalicylate.	.15	1	0	0	0	0	0	0	0	0	0	0	2+	0	0	1+	0	0	0	0	0	0
			2	0	0	0	0	0	0	0	0	0	0	2+	0	0	2+	0	0	0	0	0	0
			3	0	0	0	0	0	0	0	0	0	0	1+	0	0	2+	0	0	2+	0	0	0
			4	0	0	0	0	0	0	0	0	0	0	1+	0	0	1+	0	0	tr	0	0	0
			5	0	0	0	0	0	0	0	0	0	0	2+	0	0	3+	0	0	2+	0	0	0
6d	----------do----------	25	1	0	0	0	0	0	0	0	0	0	0	tr	0	0	0	0	0	1+	0	0	tr
			2	0	0	0	0	0	0	0	0	0	0	0	0	0	tr	0	0	2+	0	0	tr
			3	0	0	0	0	0	0	0	0	0	0	tr	0	0	tr	0	0	tr	0	0	tr
			4	0	0	0	0	0	0	0	0	0	0	0	0	0	tr	0	0	1+	0	0	tr
			5	0	0	0	0	0	0	0	0	0	0	tr	0	0	tr	0	0	1+	0	0	tr
8	2,4,5-trichlorophenol------	2.0	1	0	0	0	0	0	1+	0	2+	0	0	1+	0	0	0	0	0	2+	0	0	2+
			2	0	0	0	0	0	0	0	2+	0	0	2+	0	0	4+	0	0	2+	0	0	tr
			3	0	0	0	0	0	2+	0	0	0	0	4+	0	0	4+	0	0	2+	0	0	2+
			4	0	1+	0	0	0	0	0	0	0	0	4+	0	0	4+	0	0	tr	0	0	2+
			5	0	tr	0	2+	0	0	0	0	0	0	4+	0	0	4+	0	0	3+	0	0	2+
9	2,3,4,6-tetrachlorophenol---	5.0	1	0	tr	0	0	0	0	0	tr	0	0	3+	0	0	1+	0	0	2+	0	0	tr
			2	0	0	0	0	0	0	0	0	0	0	3+	0	0	2+	0	0	1+	0	0	0
			3	0	0	0	0	0	0	0	0	0	0	2+	0	0	2+	0	0	1+	0	0	0
			4	0	0	0	tr	0	tr	0	0	0	0	2+	0	0	1+	0	0	tr	0	0	2+
			5	0	0	0	0	0	0	0	0	0	0	3+	0	0	3+	0	0	2+	0	0	2+
12a	Paranitrophenol----------	3.0	1	0	0	0	0	0	0	0	0	0	0	4+	0	0	tr	0	0	4+	0	0	tr
			2	0	tr	0	tr	0	0	0	0	tr	0	4+	0	0	tr	tr	tr	4+	0	0	tr
			3	0	tr	0	0	0	0	0	0	tr	0	4+	0	0	tr	0	0	4+	0	0	tr
			4	0	0	0	0	0	0	0	0	tr	0	4+	0	0	tr	0	0	3+	0	0	tr
			5	0	0	0	0	0	0	0	0	0	0	4+	0	0	1+	0	0	4+	0	0	tr
12b	----------do----------	5.0	1	0	tr	0	tr	[2]0	[2]0	[2]0	[2]0	0	0	2+	0	0	tr	0	0	tr	0	0	0
			2	0	tr	0	0	0	0	0	0	0	0	tr	0	0	tr	0	0	0	0	0	0
			3	0	tr	0	tr	0	0	0	0	0	0	2+	0	0	2+	0	0	tr	0	0	0
			4	0	0	0	0	0	0	0	0	tr	0	2+	0	0	tr	0	0	2+	0	0	0
			5	0	0	0	0	0	0	0	0	0	0	2+	0	0	tr	0	0	3+	0	0	4+
14c	Parachlorometacresol--------	6.0	1	0	0	0	0	0	0	0	0	0	0	tr	0	0	tr	0	0	tr	0	0	0
			2	0	0	0	0	0	0	0	0	0	0	1+	0	0	tr	0	0	tr	0	0	0
			3	0	0	0	0	0	0	0	0	0	0	0	0	0	tr	0	0	2+	0	0	0
			4	0	0	0	0	0	0	0	0	0	0	3+	0	0	tr	0	0	tr	0	0	0
			5	0	2+	0	0	0	0	0	0	0	0	1+	0	0	2+	0	0	tr	0	0	tr
15c	Parachlorometaxylenol-------	6.0	1	0	tr	0	0	0	0	0	0	tr	0	2+	tr	0	3+	0	0	0	0	0	1+
			2	0	0	0	0	0	0	0	0	0	0	2+	0	0	4+	0	0	tr	0	0	1+
			3	0	tr	0	0	0	0	0	0	tr	0	2+	tr	0	4+	0	0	1+	0	0	2+
			4	0	tr	0	tr	0	tr	0	0	tr	0	2+	tr	0	4+	0	0	tr	0	0	1+
			5	0	0	0	0	0	0	0	0	4+	tr	3+	tr	0	4+	0	0	1+	0	0	1+
17b	2,3-dichloronaphthaquinone-1,4.	6.0	1	0	0	0	0	0	0	0	0	0	0	1+	0	0	tr	0	0	2+	0	0	2+
			2	0	0	0	0	0	0	0	0	0	0	tr	0	0	tr	0	0	2+	0	0	2+
			3	0	0	0	tr	0	tr	0	tr	0	0	tr	0	0	tr	0	0	2+	0	0	2+
			4	0	0	0	0	0	0	0	0	0	0	2+	0	0	tr	0	0	2+	0	0	2+
			5	0	0	0	tr	0	0	0	tr	0	0	1+	tr	0	tr	0	0	tr	0	0	1+

[1] Treatment numbers correspond to those used in tables 1 and 2.

[2] Reading at the end of 15 days' incubation (bottle broken).

Abbreviations: R = replicate; T = treated; U = untreated; tr = trace.

entire area was covered. The treated samples and a set of five untreated controls for each organism were inoculated by grafting on actively growing cultures of *T. interdigitale* and *E. floccosum*.

The inoculated samples were placed on glass-fabric wicks in 8-ounce square culture bottles prepared similarly to those used in previous tests, except that only 20 ml. of Sabouraud's broth was used in each of these smaller bottles. To avoid confusion in interpreting results only one leather sample was put in each bottle. The samples were incubated for 7 days, at which time observations of the presence or absence of growth of the test fungi were recorded. Each piece of leather was then transferred through a series of five tubes of Sabouraud's broth, the successive transfers being made at 24-hour intervals. The samples were left in the fifth set of tubes and all transfer tubes, from the first series through the fifth, were incubated for 15 days. Observations were made for growth at intervals of 2, 3, 5, 7, 10, and 15 days.

At the end of this time any samples not showing growth were plated on Sabouraud's agar, incubated an additional 15 days, and observed at intervals for growth. The transfer tubes in which no growth had occurred were reinoculated with 0.1 ml. of fresh suspension of the proper test fungus, then incubated for another 15 days during which time they were observed for growth. The purpose of this reinoculation was to determine whether or not sufficient chemical had leached out into the broth to prevent growth of viable cells when present.

To verify the action of each chemical tested for fungistatic and fungicidal activity against the two test organisms, further experiments using mats of fungus growth in place of inoculated leather were carried out. In this work, 0.5-cm. squares of active fungus growth on Sabouraud's agar were exposed to a solution of the chemical and then tested for viability.

The solutions of the chemicals used in these experiments with fungus mats were, with one exception, prepared in light petrolatum or mineral oil, since, when the compounds were tested on leather, they were incorporated in the oil or oil-soluble substances in the dubbing. The petrolatum was steam-sterilized at 15 pounds for 20 minutes and cooled. A definite quantity was then pipetted into a flask and enough of the desired chemical added to make the concentration in the oil correspond roughly to the weight of the compound in a 1.5- by 2.5-cm. piece of leather which had been brushed with a mixture of dubbing and

the fungicide. Since paranitrophenol does not dissolve in mineral oil in the concentrations desired, a water solution of this material was used.

To prepare the mats of fungus growth, a thin layer of Sabouraud's agar was poured into a petri dish containing 0.5 ml. of a standardized water suspension of a 5-day old fungus culture. The agar and suspension were mixed by rotating the plate, allowed to harden, then inverted and incubated for 5 days. Squares, 0.5 cm. on a side, were cut from a culture showing thick, even growth. These fungus mats were transferred, one to each of five replicate tubes in which 5 ml. of the solution of the chemical being tested had been placed, and left for 3 or 7 days--the shorter period being for chemicals showing excellent inhibiting properties. These relatively long periods of exposure were used since the organisms in leather treated with the fungicide-dubbing mixtures were considered to be in continuous contact with the chemical.

At the end of the exposure period each mat was transferred to a tube containing 10 ml. Sabouraud's broth. After 10 minutes the mats were removed to a second set of broth tubes and subsequently to third, fourth, and fifth sets of tubes at intervals of 1/2, 1/2, and 18 hours, respectively. The purpose of these transfers was to reduce the concentration of the chemical carried over on the mats from the petrolatum solution below that which would give inhibition.

Following incubation, all mats that had not shown signs of growth in the broth tubes were removed to Sabouraud's agar for another 15-day period and observed for viability. The broth tubes from which these mats had been removed were then reinoculated with a fresh, dilute suspension of the proper organism and observed for growth to see whether or not enough chemical had been carried over on the mats from the exposure tubes to inhibit the growth of viable cells.

DISCUSSION OF RESULTS

Single-Sample Tests

In all, 81 chemicals and combinations of substances were tested for inhibiting action against the growth on *Trichophyton interdigitale* and *Epidermophyton floccosum*. Of this number, 32 failed to prevent the two dermatophytes from growing when tested by modifications of the routine bacteriological procedures mentioned on page 3. The remaining 49 compounds showed vary-

ing degrees of inhibiting action in the preliminary tests. More than half of these, although fungistatic or fungicidal to some extent under certain conditions, proved ineffective when incorporated in shoe dubbing and applied to leather. However, 18 of the 49 showed moderate to excellent inhibition against the test fungi (table 1).

In the preliminary, single-sample tests 4 of the 18 chemicals--sodium ethylmercurithiosalicylate in 0.25- and 0.50-percent concentrations; 3,5-dinitro-orthocresol in a 5-percent concentration; parachlorometacresol and parachlorometaxylenol, each in a 6-percent concentration in dubbing--prevented growth of the test fungi on the treated and untreated areas when brushed only on the finished side of the leather as well as when brushed on both sides. Lower concentrations of these compounds were less effective. For example, the 0.10- and 0.20-percent concentrations of the thiosalicylate mercurial and a 5.0-percent concentration of parachlorometacresol inhibited growth of the test fungi on the treated portions of the samples but permitted them to grow on the untreated areas of a few of the pieces of leather. With a 4-percent concentration of the metacresol compound, some growth occurred even on the treated portion of the specimen. Four- and five-percent concentrations of parachlorometaxylenol were slightly less effective than the same concentrations of the similar cresol compound. Good protection was obtained from the 5-percent concentration of 3,5-dinitro-orthocresol but since it is explosive and also stains materials it touches an intense yellow, no further consideration was given to this substance.

Other compounds which, when incorporated in dubbing, inhibited growth of *T. interdigitale* and *E. floccosum* on the treated areas of the leather but not on the untreated portions were: chloride of 4-nitro-5-hydroxymercuri orthocresol anhydride (3.0 percent); phenylmercuri-9-acetoxy-12-octadecanoic acid (0.5 and 1.0 percent); 2,3,4,6-tetrachlorophenol (5.0 percent); 2,4-dinitrophenyl thiocyanate (2.0 percent)[5]; paranitrophenol (3.0 percent); hexylresorcinol (4.0 percent); and 2,3-dichloronaphthaquinone-1,4 (6.0 percent). The lower concentrations of these compounds that were tried showed even less inhibiting power. Higher concentrations of hexylresorcinol and the 4-nitro-5-hydroxymercuri orthocresol compound gave no better pro-

[5] Some cases of skin irritation caused by handling this compound have been reported. The toxicity, therefore, should be determined before using it on clothing.

TABLE 1.--*Inhibiting action against Trichophyton interdigitale and Epidermophyton floccosum of various chemicals when incorporated in shoe dubbing and applied to leather*

Treatment No.	Chemical[1]	Concentration in dubbing	*Trichophyton interdigitale*				*Epidermophyton floccosum*			
			One side		Two sides		One side		Two sides	
		Percent	*T*	*U*	*T*	*U*	*T*	*U*	*T*	*U*
1	Bismuth tribromophenate	6.0	tr	1+	0	2+	0	1+	0	1+
2a	4-nitro-5-hydroxymercuri orthocresol anhydride (chloride of)	[2]3.0	0	tr	0	1+	0	0	0	0
2b	---do---	5.0	tr	1+	0	tr	tr	2+	tr	tr
3a	Phenylmercuri-9-acetoxy-12-octadecanoic acid	.5	0	0	0	tr	0	0	0	0
3b	---do---	1.0	0	2+	0	0	0	tr	0	0
4	Phenylmercuri carbamate	.8	1+	2+	0	tr	0	0	0	tr
5	Phenylmercuric oleate	2.5	0	0	0	0	2+	4+	0	3+
6a	Sodium ethylmercurithiosalicylate	.1	0	tr	0	0	0	tr	0	tr
6b	---do---	.15	0	1+	0	0	0	0	0	0
6c	---do---	.2	0	tr	0	tr	0	0	0	0
6d	---do---	.25	0	0	0	0	0	0	0	0
6e	---do---	.5	0	0	0	0	0	0	0	0
7	Salicylanilide	5.0	0	tr	0	tr	tr	3+	0	1+
8	2,4,5-trichlorophenol	[2]2.0	0	tr	0	0	2+	4+	0	0
9	2,3,4,6-tetrachlorophenol	5.0	0	tr	0	0	0	4+	0	0
10	Crthophenylphenol	[2]5.0	0	0	0	0	tr	3+	tr	2+
11	2,4-dinitrophenyl thiocyanate[3]	2.0	0	0	0	0	0	tr	0	tr
12	Paranitrophenol	3.0	0	tr	0	tr	0	0	0	tr
13	3,5-dinitro-orthocresol	5.0	0	0	0	0	0	0	0	0
14a	Parachlorometacresol	4.0	tr	tr	0	tr	0	0	0	0
14b	---do---	5.0	0	0	0	1+	0	0	0	0
14c	---do---	6.0	0	0	0	0	0	0	0	0
15a	Parachlorometaxylenol	4.0	tr	tr	0	tr	tr	1+	0	tr
15b	---do---	5.0	tr	2+	0	2+	0	tr	0	tr
15c	---do---	6.0	0	0	0	0	0	0	0	0
16a	Hexylresorcinol	4.0	0	2+	0	1+	0	0	0	0
16b	---do---	6.0	tr	1+	0	1+	0	tr	tr	tr
17a	2,3-dichloronaphthaquinone-1,4	5.0	0	0	0	0	0	tr	tr	1+
17b	---do---	6.0	0	tr	0	tr	0	0	0	0
18	3(phenylaminomethyl) benzothiazolene-2-thione	5.0	tr	tr	0	0	0	2+	0	2+

[1] The following substances were also tested on leather in the same way but were omitted from table 1 since they showed little or no inhibiting action: (1) bismuth subsalicylate, 5 percent; (2) zinc dimethyldithiocarbamate, 2.44 percent; (3) sodium propionate, 1 percent; (4) mixture of sodium propionate and sodium undecylenate, 1 percent; (5) levulinic acid, 5 percent; (6) phenylmercuric acetate and aminoguaiacol benzothiazol immnoria, 1 percent; (7) benzyl salicylate, 5 percent; (8) benzyl orthochlorophenol, 2 percent; (9) ortho- and para-benzylphenols, 2 percent; (10) 2,2'-dihydroxy-5,5'-dichlorodiphenylmethane, 5 percent; (11) 2,2'-dihydroxy-3,3',5,5',6,6'-hexachlorodiphenylmethane, 5 percent; (12) 1,1,1-trichloro-2,2-bis(parachlorophenyl) ethane, 5 percent; (13) paratertiarvoctylphenyldiethoxy dimethyl benzylammonium oleate, 6 percent; (14) meta- and paratertiarybutylcresols, 5 percent; (15) tetrabromorthocresol, 6 percent; (16) 5-hydroxy-1,3-dimethylbenzene, 5 percent; (17) 8-hydroxyquinoline, 6 percent; (18) dichloro-8-hydroxyquinoline, 5 percent; (19) mixture of phenothiazine and orthoaminophenol, 5 percent; (20) paratoluenesulfonyl chloride, 5 percent; (21) triethanolamine, 5 percent; (22) thymol, 5 percent; (23) phenylmercuric acetate, 1 percent; (24) lauroyl colaminochloroacetate, 5 percent; (25) myristoyl colaminochloroacetate, 5 percent; (26) palmitoyl colaminochloroacetate, 5 percent; (27) stearoyl colaminochloroacetate, 5 percent; (28) monolaurin monochloroacetate; 5 percent; (29) lauryl chloroacetate, 5 percent; (30) 2-ethylhexylchloroacetate, 5 percent; and (31) colaminoformylmethylpyridinium chloride (higher acyl esters of), 5 percent.

[2] Incubated on Sabouraud's agar in place of glass wicks in Sabouraud's broth.

[3] See footnote 5, page 12.

Abbreviations: T = treated; U = untreated; tr = trace.

tection than was obtained by using 4 and 3 percent, respectively. The remaining substances listed in table 1, even in the highest concentrations used, permitted the test fungi to grow on some of the treated areas as well as on the untreated parts.

After leaching and also after storage for 2 months, six chemicals, when mixed with dubbing in the concentrations indicated, were, according to single-sample tests, completely effective in preventing growth of the two fungi when applied only to the finished side of the leather as well as when applied to both sides (table 2). They were: phenylmercuri-9-acetoxy-12-octadecanoic acid (1.0 percent); sodium ethylmercurithiosalicylate (0.15 to 0.5 percent); paranitrophenol (3.0 percent); parachlorometacresol (6 percent); parachlorometaxylenol (6 percent); and 2,3-dichloronaphthaquinone-1,4 (6 percent). In lower concentrations, four of these compounds permitted about the same growth of the test fungi on the treated samples after leaching and storage as before. The nitrophenol and the quinone derivative were not tested in lower concentrations than those indicated.

Three chemicals--bismuth tribromophenate (6.0 percent), the chloride of 4-nitro-5-hydroxymercuri orthocresol anhydride (3.0 and 5.0 percent), and hexylresorcinol (4.0 and 6.0 percent) --failed to give adequate protection when the fungicide-dubbing was brushed on only the finished side of the leather but inhibited growth when it was applied to both sides. After leaching and storing the results were practically the same as those obtained from corresponding samples tested immediately after treatment with dubbing in which these compounds had been incorporated.

Leaching and storage lowered the fungus-inhibiting ability of the 2,4-dinitrophenyl thiocyanate in the 2-percent concentration in dubbing.

Replicate Tests

The results obtained in the replicate tests on the six chemicals which, in the single-sample tests, effectively prevented growth of *T. interdigitale* and *E. floccosum* when brushed, in the dubbing mixture, on only the finished side of the leather as well as on both sides are reported in table 3. Two other chemicals 2,4,5-trichlorophenol (2 percent) and 2,3,4,6-tetrachlorophenol (5 percent), were also tested in replicate when incorporated in the dubbing even though they had not been included in the preliminary experiments.

TABLE 2.--*Effects of leaching and of storing for 2 months on the inhibiting action of various chemicals against Trichophyton interdigitale and Epidermophyton floccosum when incorporated in shoe dubbing and applied to leather*

Treatment No.[1]	Chemical	Concentrations in dubbing	Leaching test						Storage test					
			Trichophyton interdigitale			*Epidermophyton floccosum*			*Trichophyton interdigitale*			*Epidermophyton floccosum*		
			One side	Two sides	Untreated controls	One side	Two sides	Untreated controls	One side	Two sides	Untreated controls	One side	Two sides	Untreated controls
		Percent												
1	Bismuth tribromophenate	6.0	tr	0	3+	tr	0	3+	2+	0	3+	2+	0	3+
2a	4-nitro-5-hydroxymercuri orthocresol anhydride (chloride of)	[2]3.0	tr	0	2+	tr	0	4+	tr	0	3+	0	0	1+
2b	---do---	5.0	2+	0	4+	tr	0	4+	tr	0	3+	2+	0	3+
3a	Phenylmercuri-9-acetoxy-12-octadecanoic acid	.5	tr	0	4+	3+	0	4+	3+	0	4+	tr	0	3+
3b	---do---	1.0	0	0	4+	0	0	0	0	0	3+	0	0	3+
6a	Sodium ethylmercurithiosalicylate	.1	tr	tr	4+	0	0	2+	tr	0	2+	0	0	tr
6b	---do---	.15	0	0	tr	0	0	[3]0	0	0	2+	0	0	tr
6c	---do---	.2	0	0	2+	0	0	tr	0	0	2+	0	0	0
6d	---do---	.25	0	0	[3]0	0	0	[3]0	0	0	tr	0	0	tr
6e	---do---	.5	0	0	[3]0	0	0	[3]0	0	0	[3]0	0	0	tr
11	2,4-dinitrophenyl thiocyanate[4]	2.0	0	0	3+	tr	tr	3+	tr	tr	4+	tr	0	3+
12	Paranitrophenol	3.0	0	0	2+	0	0	1+	0	0	tr	0	0	tr
14a	Parachlorometacresol	4.0	2+	tr	2+	tr	0	4+	tr	0	2+	0	0	tr
14c	---do---	6.0	0	0	tr	0	0	tr	0	0	2+	0	0	3+
15b	Parachlorometaxylenol	5.0	tr	0	tr	tr	0	3+	1+	0	4+	tr	0	3+
15c	---do---	6.0	0	0	tr	0	0	2+	tr	0	4+	0	0	3+
16a	Hexylresorcinol	4.0	tr	0	4+	0	0	4+	tr	0	2+	tr	0	2+
16b	---do---	6.0	tr	0	4+	tr	0	3+	tr	0	3+	0	0	3+
17b	2,3-dichloronaphthaquinone-1,4	6.0	0	0	2+	0	0	2+	0	0	4+	0	0	3+

[1] Treatment numbers correspond to those used in table 1 for the same chemical dubbing combinations.

[2] Incubated on Sabouraud's agar in place of glass wicks in Sabouraud's broth.

[3] These controls were inoculated on the same culture plates as some of the treated samples and probably sufficient chemical diffused out to cause inhibition on the controls.

[4] See footnote 5, page 12.

Abbreviation: tr = trace.

Of the eight chemicals studied in replicate, sodium ethylmercurithiosalicylate gave outstandingly good results. It killed both dermatophytes in much lower concentrations in dubbing (0.15 and 0.25 percent) than were required for fungicidal activity of any other compound used. It did not leach out during the five water rinses extending over a period of 31 hours and did not lose its fungicidal activity after 2 months' storage in the dark. It was the only compound that diffused sufficiently into the untreated parts of the leather to prevent completely the growth of the test fungi on those areas.

Since sodium ethylmercurithiosalicylate is water soluble as well as readily compatible with the dubbing and since only very small amounts of it are necessary to prevent growth of the test organisms, there may have been adequate moisture in the form of the broth medium around the leather samples during incubation to draw enough of the chemical into the untreated portions of the leather to cause inhibition.

The other seven compounds studied in replicate (table 3) completely prevented *T. interdigitale* and *E. floccosum* from growing on the treated portions of the samples but did not diffuse into the untreated areas as effectively as did the sodium ethylmercurithiosalicylate. The action of parachlorometacresol, 2,4,5-trichlorophenol, and 2,3,4,6-tetrachlorophenol in 6.0-, 2.0-, and 5.0-percent concentrations, respectively, which completely inhibited the fungus growth on treated areas, was unaffected by leaching and by storage. These chemicals were equally effective against *Trichophyton* and *Epidermophyton*.

Paranitrophenol, in the 5-percent solution, was completely inhibitory against *E. floccosum* but leached out sufficiently to permit slight growth of *T. interdigitale* on some of the replicates. In the 3-percent concentration, leaching and storage both lessened the effectiveness of this compound in inhibiting growth of *Trichophyton*.

Phenylmercuri-9-acetoxy-12-octadecanoic acid, completely inhibitory in a 2-percent concentration against both these organisms on the treated areas of all samples, leached out enough to allow *Epidermophyton* to grow on one of the replicates brushed on only one side. It also failed to prevent growth of the same fungus on a similarly treated sample stored for 2 months.

A 6-percent concentration of 2,3-dichloronaphthaquinone-1,4 in dubbing protected the treated portions and was unaffected by storage. However, leaching lessened the effectiveness of this treatment so that *Epidermophyton* grew on one of the pieces brushed on only the finished side.

Parachlorometaxylenol (6.0 percent) gave good protection against the two fungi on the treated areas of the leather samples, and was equally effective after storage as before. However, this chemical was affected much more by the 31-hour soaking in water than were any of the other substances. Leaching removed enough of the metaxylenol to allow growth of both organisms on most of the samples brushed only on the finished side and of the *Trichophyton* on one of the replicates treated on both sides.

Fungicidal-Fungistatic Action

In the concentrations studied, sodium ethylmercurithiosalicylate was the only compound found to be fungicidal to both organisms. Paranitrophenol, at the 3-percent level, was fungicidal against *Epidermophyton* and fungistatic to *Trichophyton*. The 5-percent concentration of this chemical was not used in the fungicidal-fungistatic tests. The 2,3,4,6-tetrachlorophenol and 2,3-dichloronaphthaquinone-1,4 in 5- and 6-percent concentrations, respectively, were fungicidal for *Trichophyton* and fungistatic for *Epidermophyton*. The 6-percent concentrations of parachlorometacresol and parachlorometaxylenol and the 2-percent solution of 2,4,5-trichlorophenol inhibited both fungi but failed to kill either on all of the replicate samples.

CONCLUSIONS

Growth of the two dermatophytes, *Trichophyton interdigitale* and *Epidermophyton floccosum*, in chrome-retanned leather, such as that used for the uppers of shoes, could be inhibited by incorporating certain chemicals in the dressing applied to leather. In some cases the inhibiting action was fungicidal; in others, fungistatic. Eight of the substances investigated were good to excellent inhibitors of the growth of these fungi.

Four of the eight chemicals preventing growth of *Trichophyton* and *Epidermophyton* on leather when incorporated in dubbing were as effective after leaching as before and six retained their full inhibiting action during 2 months of storage. In the other instances the ability of the chemical dubbing mixtures in preventing growth of the test fungi was lessened to varying degrees by leaching and by storage.

Although several of the chemicals were satisfactory fungicides from a mycological standpoint, their toxicity should be tested before they are used in the control or prevention of infections caused by the two dermatophytes, *T. interdigitale* and *E. floccosum*.

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